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Origin plasticity during budding yeast DNA replication *in vitro*

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 09 December 2013

Thank you again for submitting your manuscript on cell-free budding yeast replication for our editorial consideration. We have now received the comments of three expert referees, which you will find copied below for your information. I am happy to inform you that all three referees consider this work in principle of interest and importance, and therefore suitable for publication pending satisfactory addressing of a limited number of specific concerns raised in all three reports. I would therefore like to invite you to revise the manuscript along the lines suggested by the referees, keeping in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully respond to all points at this stage. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1:

The initiation of chromosomal DNA replication in eukaryotic cells consists of consecutive two steps; formation of the pre-replicative complex (pre-RC) in the absence of cyclin-dependent protein kinase (CDK) and assembly of replication proteins on origins in the presence of CDK and Dbf4-dependent protein kinase (DDK). Recent development of in vitro replication system using budding yeast extract and reconstitution of the pre-RC from its purified components devised the way to investigate the initiation step of eukaryotic DNA replication in detail. However, it had been unknown whether the reconstituted pre-RC is functional for initiation of DNA replication. In this manuscript the authors showed that the reconstituted pre-RC is functional in in vitro replication system. Moreover, they find that origin specificity is low and CEN4 sequence is preferred by ORC and then for initiation as well as ARS1 in their conditions. Furthermore, they suggested that efficiency of initiation depends on DNA sequence context. In addition, the authors analyzed the product quite extensively and found that DNA molecule is fully replicated in the bead-free system. The experiments were carried out carefully and well documented. The results shown in the manuscript support their conclusion.
The manuscript enables us considering the knowledge of the reconstituted pre-RC reported here and before as a biologically functional pre-RC and also provides the tool to improve the in vitro replication system. Instead, low specificity of ORC was described previously and low origin-specific firing is expected. Novelty here is that efficiency of initiation of replication depends on DNA-sequence context as the authors suggested in Fig. 9.
Taken altogether, this manuscript advances our knowledge of eukaryotic DNA replication significantly and opens the way to analyze the initiation step in detail. I thus support publication of this manuscript with slight modification.

(Specific points)
1. p.6, line 15, the references lack Kamimura et al., EMBO J 20, 2097-2107, 2001.
2. Figure 2E. Mbo1 and Sau3A digestions will be appreciated as controls.

Referee #2:

Gros et al. describes an assay that replicates DNA in vitro with two distinguishing features from a previous yeast DNA replication assay: 1) the reconstitution of pre-RC formation with purified components and 2) the use of a plasmid unbound from beads. Their data show replication results in covalently closed circular products that are dependent on DDK, CDK, and the components of the pre-RC providing verification of many replication hallmarks previously seen in vivo. In addition, they show that a number of observations made in the initial description of the origin-dependent replication assay are also true in this system. The authors subsequently show ARS elements, which are essential in vivo, are not essential in this in vitro replication system.

This paper makes a strong case that ORC binding to non-ARS sequences can initiate replication. Previous in vivo data would suggest an ARS1 A-B2- mutant should not be competent to initiate replication. Strikingly, the authors show ATP-dependent binding of ORC to regions of a plasmid lacking an ARS element. These data suggest ORC is redistributed to other sites on a plasmid with a mutated ARS, and pre-RCs formed at these sites are able to replicate DNA. It raises interesting questions about the accessibility of DNA and the mechanism of origin selection during cellular replication. It is noteworthy, however, that it is not clear that ORC binding to other sites in the plasmid is fundamentally different than ORC binding at known ARS elements and the results obtained could be due to the known low specificity of ORC DNA binding.

The authors also suggest that Mcm2-7 loading is not sufficient for subsequent helicase activation and replication elongation and that there is a role of specific DNA sequences after helicase loading. The strongest evidence in favor of this conclusion is that for plasmids containing wild-type or mutant ARS305, there is reduced replication for the mutant plasmid (Fig. 7A) even though the amount of Mcm2-7 loaded on the DNA is the same (Fig. 7B). Similarly, the authors observe similar amounts of Mcm2-7 loading on a bacterial plasmid and the same plasmid with an origin but very different replication levels for the same templates. Despite this evidence, there are several concerns about these conclusions. First, the conditions used for pre-RC formation in the replication assay are different than the conditions used for the pre-RC formation-only assays. Since the authors see less

specificity for pre-RC formation than replication, the increased protein concentrations in the pre-RC assays could be responsible for the different specificity observed. Second, the authors report that high-salt treatment does not effect the replication assay, however, Mcm2-7 is known to slide along DNA after this treatment. This observation suggests Mcm2-7 is competent to be activated independent of the associated DNA sequence (i.e. sliding away from the origin does not inhibit initiation). Third, when the authors use a different IP-based assay for Mcm2-7 loading in figure 8, they suggest that there are similar extents of loading for the wild-type and mutant ARS305 plasmids. However, the total amount of A- pARS305 DNA pulled down in figure 8C does not appear to be equal to the total amount of WT pARS305 DNA. Quantification of the data should be included and if there are differences they should be discussed.

Overall, the data presented further refines the approaches to investigate replication initiation and has the potential to provide new insights into the specificity of these events.

Specific points:

1. A better explanation is needed for the calculations used to determine the extent of replication in the in vitro system. It was not clear how the 1-2% was calculated from figure 2E. Since the number of pre-RCs loaded in not quantified, it would be better to state that an unknown step before elongation is likely the reason for the low levels of replication.

2. Although it important to show replication is dependent on each component of the pre-RC to be present, the explanation of why was misleading. The endogenous pre-RC components in the S phase extract are unlikely to be functional when combined with the purified components since we know CDK inhibits pre-RC formation. A more logical argument would emphasize strand displacement synthesis or an alternative (origin-independent) method of replication initiation upon addition of the S phase extract.

3. The first of the two models for ARS-independent replication needs to be stated more clearly. Is the model proposing pre-RCs can load at infinite locations on pARS1 or at several defined locations at similar levels? This model could be separated into two distinct models as well.

Referee #3:

Eukaryotic DNA replication initiates from multiple origins of replication. In buddying yeast origins are well-defined by specific DNA sequences which support origin recognition complex (ORC) binding and loading of inactive core of replicative helicase (Mcm2-7). During replication initiation this inactive helicase is remodelled and activated due to action of S-phase kinases and binding of additional factors. As DNA replication is one of the most fundamental processes in life it is highly conserved through evolution and thus often studied in simpler eukaryotes or model systems. Much of what is known about this process comes from studies in buddying and fission yeast and cell free system of Xenopus laevis egg extract. The last 5 years have seen an explosion of a new model system in the replication field - the buddying yeast cell free reconstitution system. The first step of replication - the loading of the inactive helicase onto exogenous DNA (pre-RC establishment) has been reconstituted previously with both G1 arrested cell extract and with purified proteins, and the extract loaded helicase has also been shown to be able to be activated by S-phase arrested extract. The present manuscript takes this system a step further. Gros et.al. established a cell free system able to fully, and in a semiconservative manner, replicate plasmids on which pre-RC are loaded by a defined set of purified proteins. The observed replication, although not very efficient, is mostly governed by the same requirements as replication in vivo and replication of plasmids with pre-RCs assembled by cell extract (Heller et. al. 2011), indicating that double-hexamers of Mcm2-7, reconstituted with purified proteins, are indeed intermediates of replication initiation reaction. Pre-RCs reconstituted with purified proteins are dependent on the presence of the buddying yeast origin sequence only when challenged with a DNA competitor (Remus et al 2009). Similarly here, and unlike in vivo, replication of plasmids often does not depend on known replication origin DNA sequences, with many heterologous sequences able to assemble pre-RCs and replicate. Importantly, however, not every plasmid can replicate in this system suggesting the importance of some sequence features. Moreover, intriguingly, the ability to assemble pre-RCs on plasmid does not guarantee replication, suggesting that specific sequence features may also be important for replication

initiation past the pre-RC formation.

Major points:

1. Figure 1c. Authors suggest that no accumulation of RPA after aphidicolin treatment suggests the lack of long stretches of DNA after the DNA synthesis inhibition. It is possible, however, that there are many fewer pre-ICs transformed into active forks under these conditions. RPA is the only non-overexpressed protein tested and so it is difficult to judge this. Could the authors blot for GINS or other replisome components to compare their levels? It is possible also that due to the attachment of plasmid DNA to the beads the helicase ability to run off upon aphidicolin treatment is strongly reduced.
2. Why do some alkaline gels show clear two bands (ssl and ccc) while others do not? (Fig 2A and C or Fig 3A and B).
3. Mcm10 is clearly essential for DNA replication as shown here and before, but authors suggest also that "immuno-depletion of Mcm10 from the S phase extract, while greatly reducing DNA synthesis activity, did not significantly affect the recruitment of Cdc45, Sld3, or Dpb11 (Supplementary Figure S2A-C) supporting earlier observations that Mcm10 acts after pre-IC and CMG assembly". Fig S2C shows about 50% reduction of Cdc45 binding after Mcm10 depletion - is this effect reproducible? Is it the same for GINS? Potentially Mcm10 depletion could reduce GINS loading more severely as, unlike Cdc45, GINS is not overexpressed in this system.
4. Addition of rMcm10 to Mcm10 depleted extract rescues and indeed slightly increases the level of replicated DNA (Fig 3A). Is this reproducible? How much recombinant Mcm10 has been added to the extract - is it close to the endogenous Mcm10 levels? Would DNA replication efficiency increase with more Mcm10 added? Is Mcm10 a limiting factor for efficient replication in this system?
5. The authors speculate that an as yet unknown aspect of DNA sequence may contribute to efficient origin function, most likely through origin melting regulation. Are pre-ICs established as well on pET16b pre-RCs as on pARS1 pre-RCs? Is there a difference in Mcm10 loading? Knowing these could strongly support the authors' speculation.

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5. On p. 14 authors say: "pre-RCs assembled outside ARS305 in pARS305 appeared to get activated less efficiently", although they have not shown it per se. There is less or no replication driven by these pre-RCs but it can also be explained by other defects - i.e. a defect in the elongation stage.

1st Revision - authors' response 13 December 2013

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that DNA molecule is fully replicated in the bead-free system. The experiments were carried out carefully and well documented. The results shown in the manuscript support their conclusion.
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This reference has now been included.

2. Figure 2E. Mbo1 and Sau3A digestions will be appreciated as controls.

This experiment is included in the revised manuscript as new supplementary Figure S6.

Referee #2:

Gros et al. describes an assay that replicates DNA in vitro with two distinguishing features from a previous yeast DNA replication assay: 1) the reconstitution of pre-RC formation with purified components and 2) the use of a plasmid unbound from beads. Their data show replication results in covalently closed circular products that are dependent on DDK, CDK, and the components of the pre-RC providing verification of many replication hallmarks previously seen in vivo. In addition, they show that a number of observations made in the initial description of the origin-dependent replication assay are also true in this system. The authors subsequently show ARS elements, which are essential in vivo, are not essential in this in vitro replication system.

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The authors also suggest that Mcm2-7 loading is not sufficient for subsequent helicase activation and replication elongation and that there is a role of specific DNA sequences after helicase loading. The strongest evidence in favor of this conclusion is that for plasmids containing wild-type or mutant ARS305, there is reduced replication for the mutant plasmid (Fig. 7A) even though the amount of Mcm2-7 loaded on the DNA is the same (Fig. 7B). Similarly, the authors observe similar amounts of Mcm2-7 loading on a bacterial plasmid and the same plasmid with an origin but very different replication levels for the same templates. Despite this evidence, there are several concerns about these conclusions.

First, the conditions used for pre-RC formation in the replication assay are different than the conditions used for the pre-RC formation-only assays. Since the authors see less specificity for pre-RC formation than replication, the increased protein concentrations in the pre-RC assays could be responsible for the different specificity observed.

We agree with the reviewer that differences in protein concentrations would affect the specificity of pre-RC assembly. In agreement with this notion we demonstrate in figures 5 and 8 that increasing concentrations of ORC alone in the presence of constant concentrations of Cdc6 and Cdt1·Mcm2-7 result in increasingly promiscuous pre-RC assembly. For this reason pre-RC assembly in figures 7B

and 9D was performed at limiting ORC concentrations of 10 nM. While we had indicated this in the figure legend of figure 9D, we had inadvertently omitted this information from the figure legend of figure 7B; this information has now been included in figure legend 7B. Cdc6 and Cdt1·Mcm2-7 concentrations, as well as input amount of template plasmid DNA, and buffer conditions were otherwise identical to those used during *in vitro* DNA replication.

Second, the authors report that high-salt treatment does not effect the replication assay, however, Mcm2-7 is known to slide along DNA after this treatment. This observation suggests Mcm2-7 is competent to be activated independent of the associated DNA sequence (i.e. sliding away from the origin does not inhibit initiation).

We agree that one implication of the experiment in Figure 1B is that Mcm2-7 remain competent to get activated after high-salt-induced sliding. We have actually discussed this in the original manuscript (pages 5 and 6) and, therefore, feel that we do not have to further address this issue.

Third, when the authors use a different IP-based assay for Mcm2-7 loading in figure 8, they suggest that there are similar extents of loading for the wild-type and mutant ARS305 plasmids. However, the total amount of A- pARS305 DNA pulled down in figure 8C does not appear to be equal to the total amount of WT pARS305 DNA. Quantification of the data should be included and if there are differences they should be discussed.

We have now included a quantification of the amount of DNA (as determined by phosphorimager analysis) associated with Mcm2-7 in each lane of Figure 8C. The data show that total Mcm2-7 loading on pARS305 plasmid is not significantly reduced upon mutation of the essential ACS in ARS305. Moreover, quantification of the relative amounts of 2.7 kb and 1.9 kb fragment co-immuno-precipitated with Mcm3 shows that these fragments are recovered at proportionally increased levels in the A- mutant plasmid compared to the wt plasmid (not shown). Together, these data support our model that Mcm2-7 loading sites are redistributed from ARS305 upon mutational inactivation of ARS305.

Overall, the data presented further refines the approaches to investigate replication initiation and has the potential to provide new insights into the specificity of these events.

Specific points:

1. A better explanation is needed for the calculations used to determine the extent of replication in the in vitro system. It was not clear how the 1-2% was calculated from figure 2E. Since the number of pre-RCs loaded in not quantified, it would be better to state that an unknown step before elongation is likely the reason for the low levels of replication.

As stated in the main text of the original manuscript, the 1-2% replication efficiency was estimated from the Dpn1-resistant fraction of template DNA molecules as determined after agarose gel-electrophoresis and ethidium-bromide staining of the DNA as in Figure 2A (lanes 1-3). We agree that this method only allows for a rough estimation and have, therefore, decided to replace this estimate with the statement that "only a small fraction of the input DNA molecules is replicated in our system". We agree with the reviewer that a step preceding elongation is likely to limit the replication efficiency of the system and have stated this more clearly in the main text (page 9)

2. Although it important to show replication is dependent on each component of the pre-RC to be present, the explanation of why was misleading. The endogenous pre-RC components in the S phase extract are unlikely to be functional when combined with the purified components since we know CDK inhibits pre-RC formation. A more logical argument would emphasize strand displacement synthesis or an alternative (origin-independent) method of replication initiation upon addition of the S phase extract.

We fully agree with the reviewer, but would like to point out that we had discussed both the CDK-inhibition of pre-RC components in the extract and potential strand-displacement synthesis in the original manuscript (pages 9 and 10). Moreover, we consider it important to test the possibility of complex formation between endogenous and purified pre-RC components as some pre-RC components may conceivably lose their inhibitory phosphorylation due to unregulated phosphatase

activity in the whole-cell extract. We appreciate, however, the suggestion to consider the possibility of alternative origin-independent initiation mechanisms and now raise this point in the revised main text.

3. The first of the two models for ARS-independent replication needs to be stated more clearly. Is the model proposing pre-RCs can load at infinite locations on pARS1 or at several defined locations at similar levels? This model could be separated into two distinct models as well.

We thank the reviewer for pointing out this shortcoming of our first model and for the suggestion to separate model one into two distinct models. We have revised our manuscript accordingly.

Referee #3:

Eukaryotic DNA replication initiates from multiple origins of replication. In buddying yeast origins are well-defined by specific DNA sequences which support origin recognition complex (ORC) binding and loading of inactive core of replicative helicase (Mcm2-7). During replication initiation this inactive helicase is remodelled and activated due to action of S-phase kinases and binding of additional factors. As DNA replication is one of the most fundamental processes in life it is highly conserved through evolution and thus often studied in simpler eukaryotes or model systems. Much of what is known about this process comes from studies in buddying and fission yeast and cell free system of Xenopus laevis egg extract. The last 5 years have seen an explosion of a new model system in the replication field - the buddying yeast cell free reconstitution system. The first step of replication - the loading of the inactive helicase onto exogenous DNA (pre-RC establishment) has been
reconstituted previously with both G1 arrested cell extract and with purified proteins, and the extract loaded helicase has also been shown to be able to be activated by S-phase arrested extract. The present manuscript takes this system a step further. Gros et.al. established a cell free system able to fully, and in a semiconservative manner, replicate plasmids on which pre-RC are loaded by a defined set of purified proteins. The observed replication, although not very efficient, is mostly governed by the same requirements as replication in vivo and replication of plasmids with pre-RCs assembled by cell extract (Heller et. al. 2011), indicating that double-hexamers of Mcm2-7, reconstituted with purified proteins, are indeed intermediates of replication initiation reaction. Pre-RCs reconstituted with purified proteins are dependent on the presence of the buddying yeast origin sequence only when challenged with a DNA competitor (Remus et al 2009). Similarly here, and unlike in vivo, replication of plasmids often does not depend on known replication origin DNA sequences, with many heterologous sequences able to assemble pre-RCs and replicate. Importantly, however, not every plasmid can replicate in this system suggesting the importance of some sequence features. Moreover, intriguingly, the ability to assemble pre-RCs on plasmid does not guarantee replication, suggesting that specific sequence features may also be important for replication initiation past the pre-RC formation.

Major points:

1. Figure 1c. Authors suggest that no accumulation of RPA after aphidicolin treatment suggests the lack of long stretches of DNA after the DNA synthesis inhibition. It is possible, however, that there are many fewer pre-ICs transformed into active forks under these conditions. RPA is the only non-overexpressed protein tested and so it is difficult to judge this. Could the authors blot for GINS or other replisome components to compare their levels? It is possible also that due to the attachment of plasmid DNA to the beads the helicase ability to run off upon aphidicolin treatment is strongly reduced.

We show that loading of Cdc45, Sld2, and Sld3, are essentially unaffected by the presence of aphidicolin in the extract (Figure 1C, compare lanes 4 and 5), suggesting that pre-IC assembly is not grossly affected by aphidicolin. Although RPA is not overproduced here, RPA is abundant in the extract (our own observations) and we, therefore, consider it not to be limiting. More importantly, we observe that RPA association with the DNA template is actually reduced in the presence of aphidicolin (Figure 1C, lanes 4 and 5), demonstrating that limiting RPA levels are not the reason for lack of accumulation in the presence of aphidicolin. Coupling of the template DNA to beads is

unlikely to prevent the generation of large unwound sections of plasmid DNA, as nascent DNA strands gain lengths of several kilobases during *in vitro* replication on beads (Figure 1), demonstrating that several thousand basepairs of DNA can principally be unwound also on beads. Unfortunately we do not have currently an antibody available to detect GINS by Western blot.

2. Why do some alkaline gels show clear two bands (ssl and ccc) while others do not? (Fig 2A and C or Fig 3A and B).

Although we standardly observe both ssl and ccc DNA species on alkaline gels, we agree with the reviewer and have also stated in the manuscript that those two species are not always evident on our alkaline agarose gels. We do not have a solid explanation for this, but we think it is most likely a result of variation in sample preparation due to different lab members performing the experiments. Importantly, the ability to distinguish between these two species is not critical here and does not affect any of the conclusions presented here.

3. Mcm10 is clearly essential for DNA replication as shown here and before, but authors suggest also that "immuno-depletion of Mcm10 from the S phase extract, while greatly reducing DNA synthesis activity, did not significantly affect the recruitment of Cdc45, Sld3, or Dpb11 (Supplementary Figure S2A-C) supporting earlier observations that Mcm10 acts after pre-IC and CMG assembly". Fig S2C shows about 50% reduction of Cdc45 binding after Mcm10 depletion - is this effect reproducible? Is it the same for GINS? Potentially Mcm10 depletion could reduce GINS loading more severely as, unlike Cdc45, GINS is not overexpressed in this system.

We agree with the reviewer that it would be important to determine the effects on the assembly of all known pre-IC components in order to determine conclusively if pre-IC assembly is affected by the absence of Mcm10. We, therefore, prefer to state that our results 'suggest' that pre-IC assembly is not grossly affected. Due to the unavailability of antibodies we have unfortunately not been able to follow GINS association. We note, however, that we attempt to always monitor both pathways essential for pre-IC assembly, i.e. DDK-dependent (Sld3 and Cdc45 association) and CDK-dependent (Sld2 and Dpb11 association) steps. We furthermore note that we perform the pre-IC assembly reactions in the absence of cross-linking agent (which is otherwise required, for example, to observe Sld3 or Cdc45 binding in G1 phase in the absence of CDK activity), which may introduce variability in the efficiency of binding (although this is generally not observed by us). We, therefore, now say in the revised manuscript that Mcm10 does 'not eliminate' instead of 'not affect' Sld3, Cdc45, or Dpb11 binding, to reflect the possibility that Mcm10 may have more subtle effects on pre-IC assembly that cannot be excluded at this point.

4. Addition of rMcm10 to Mcm10 depleted extract rescues and indeed slightly increases the level of replicated DNA (Fig 3A). Is this reproducible? How much recombinant Mcm10 has been added to the extract - is it close to the endogenous Mcm10 levels? Would DNA replication efficiency increase with more Mcm10 added? Is Mcm10 a limiting factor for efficient replication in this system?

We use a myc epitope tag to detect endogenous Mcm10 in the extract, but rescue the depletion with untagged recombinant Mcm10. Moreover, we do not have an assay that would allow us to determine the active fraction of Mcm10 in our recombinant protein preparation. It is therefore difficult for us to determine the effective relative level of recombinant Mcm10 used for the rescue compared to the endogenous protein present in the extract. We note, however, that we can rescue the replication defect of Mcm10-depleted extracts by adding only 100 fmol (1.25 nM final) of recombinant Mcm10, as stated in the supplementary materials and methods. We have empirically determined the amount of Mcm10 required for rescue and have found that Mcm10 needs to be carefully titrated, as higher amounts of Mcm10 actually inhibit the DNA replication reaction (for reasons that we do not know). This data can be made available to the reviewer upon request. As such Mcm10 thus seems not to be limiting under the conditions used here.

5. The authors speculate that an as yet unknown aspect of DNA sequence may contribute to efficient origin function, most likely through origin melting regulation. Are pre-ICs established as well on pET16b pre-RCs as on pARS1 pre-RCs? Is there a difference in Mcm10 loading? Knowing these could strongly support the authors' speculation.

We agree with the reviewer that it will be interesting and important to determine the step in the

replication reaction that is inhibited after pre-RC assembly on replication-deficient DNA templates. However, we feel that such an analysis is beyond the scope of the current study. We acknowledge the reviewer's suggestion that a step other than melting may also be affected by the DNA sequences bound to Mcm2-7 and have amended our hypotheses to include the possibility that replisome assembly, in addition to DNA melting, may depend on DNA sequence context.

Minor points:

1. DNA gels in Figures 2C and D, 4A, 9B and E, S2B, S3, S5, S7 are lacking size markers

Size markers have been added to the respective figures.

2. Authors should cite 'Cuvier et al 2008 Genes and Dev' paper showing ICRF-193 blocking termination of DNA replication when discussing ICRF-193

We have included a reference to this study in the revised manuscript.

3. Do pET16b and pARS305-A- contain fewer matches to ACS within their sequence than pARS1 or pBluescript?

We have analyzed the plasmids used in this study for the presence of sequences containing 0, 1, or 2 mismatches to the ACS. However, it is important to note that imperfect matches to the ACS may be better ORC binding sites than perfect ACS matches, as demonstrated by our example of CEN4, where ORC prefers to bind to an imperfect ACS over a nearby perfect ACS (see figure 6). At lower stringency (1-2 mismatches allowed), all plasmids tested here contain multiple imperfect matches to the ACS, and it appears that the number of ACSs is roughly proportional to the size of the plasmid. pARS305 (both wt and A- mutant), contains a greater number of ACS matches than pARS1, and both pARS1 and pARS305 contain more potential matches than either pBluescript or pET16b. But as it is currently impossible to predict "high-affinity" and "low-affinity" ACSs based on sequence information alone we feel opposed to trying to attempt to correlate the number of ACSs with replication competency.

4. Are results in Figures 6c, 7a, 9c, 9f reproducible? Could the authors put error bars on them as in 9g?

All experiments presented here are reproducible. As indicated in the figure legends for figures 6C and 7A, graphs depict average values with error bars too small to be depicted in the graph. Figures 9C and 9F are quantifications of representative experiments. In these cases, experiments were performed multiple times under slightly varying conditions (varying ORC concentrations, plasmid concentrations, time points), such that individual data points could not be directly compared. However, as the results with any given template are qualitatively similar under different conditions, we feel that showing representative experiments is adequate.

5. On p. 14 authors say: "pre-RCs assembled outside ARS305 in pARS305 appeared to get activated less efficiently", although they have not shown it per se. There is less or no replication driven by these pre-RCs but it can also be explained by other defects - i.e. a defect in the elongation stage.

We disagree with the reviewer on this interpretation, as a defect in elongation would result in shorter nascent strands over time. In contrast, we do not observe short replication products on replication-deficient templates, but instead find that nascent strands derived from pARS305-A- replication *in vitro* are indistinguishable from those of the wildtype plasmid (see gel figure 7), but strongly reduced in terms of overall levels, consistent with an initiation defect. Similarly, replication products for pET16b contain full-length replicated plasmid monomers, but no significantly higher fraction of partially replicated molecules (figure 9E).

Acceptance letter 10 January 2014

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before proceeding with formal acceptance and transfer to production, I only need to ask you to introduce the qualifier statement into the text as suggested by the referee, and email a modified text document to us.
After that, we should then be able to swiftly proceed with formal acceptance and publication of the manuscript!

Referee #2

The revised manuscript is appropriate for publication with one minor modification. When the authors describe figure 8C, they should add a qualifier to the statement that the amount of DNA is "unchanged" between the wild-type and mutant plasmids. Since there is a drop of 7%, (100% to 93%), the authors should change there statement to match these numbers (e.g." is similar").

